In the Sequence Listing:

Please delete the Sequence Listing filed January 22, 2004, and substitute the Sequence Listing filed herewith therefor. Some sequences disclosed in the Specification and/or Drawings of the originally filed application were not originally assigned sequence identifiers, and therefore the Sequence Listing and Specification have been amended herein to include such sequence identifiers. Applicants respectfully submit that the Sequence Listing filed herewith does not contain new matter. Entry thereof is respectfully requested.

Amendments to the Specification:

Please amend the Specification as follows. Applicant respectfully submits that such amendments are simply corrections required in response to the Examiner's objections to the disclosure or addition of sequence identifiers to previously disclosed sequences, and therefore such amendments do not constitute new matter. Entry thereof is respectfully requested.

[0016] Some of the current methods for designing and constructing carbohydrate polymers *in vitro* utilize: (i) difficult, multistep sugar chemistry, or (ii) reactions driven by transferase enzymes involved in biosynthesis, or (iii) reactions harnessing carbohydrate degrading enzymes catalyzing transglycosylation or hydrolysis. The latter two methods are often restricted by the specificity and the properties of the available naturally occurring enzymes. Many of these enzymes are neither particularly abundant nor stable but are almost always expensive. Overall, the procedures currently employed yield polymers containing between 2 and about 12 sugars. Unfortunately, many of the physical and biological properties of polysaccharides do not become apparent until the polymer contains ~~25, 100,~~ **25-100** or even thousands of monomers.

[0053] FIG. 7 is a pictorial representation of the pmHAS truncation mutants. **N (1-972) is SEQ ID NO:1; A (437-972) is SEQ ID NO:13; B (437-756) is SEQ ID NO:14; C (1-756) is SEQ ID NO:20; D (1-703) is SEQ ID NO:71; E (1-650) is SEQ ID NO:10; F (1-567) is SEQ ID NO:21; and G (152-756) is SEQ ID NO:15.**

[0055] FIG. 9 is a Western Blot analysis showing the expression of pmHAS and its truncated forms. Either whole cell lysates ($pmHAS^{437-972}$ **(SEQ ID NO:13)**, $pmHAS^{1-567}$ **(SEQ ID NO:21)**, and $pmHAS^{152-756}$ **(SEQ ID NO:15)**) or membrane preparations ($pmHAS^{437-756}$ **(SEQ ID NO:14)**, $pmHAS^{1-567}$ **(SEQ ID NO:21)**, **r**1-972 **(SEQ ID NO:1)**, **n**1-972 **(SEQ ID NO:1)**) or B-Per extract ($pmHAS^{1-703}$ **(SEQ ID NO:71)**) were analyzed by Western blot (**r**,recombinant from *E. coli*; **n**, native from P-1059). The *bars* on the left denote the position of molecular weight standards (from top to bottom: 112, 95, 55, and 29 kDa).

[0056] FIG. 10 is a pictorial representation of domains A1 **(SEQ ID NO:72)** and A2 **(SEQ ID NO:73)** of pmHAS. **(A)** The approximate relative positions of domain A1 and A2 in pmHAS and $pmHAS^{1-703}$. **(B)** Partial alignment of the amino acid sequences of the two domains (residue 161-267 and 443-547). The aspartate residues mutated in our studies were marked with *. Identical residues are in **bold**.

[0057] FIG. 11 is a graphical representation of the complementation of the HAS activity of mutant enzymes *in vitro*. HAS enzyme assays with HA-derived acceptor were performed in the presence of either wild type pmHAS$^{1-703}$ **(SEQ ID NO:71)** alone, or D196**N** mutant **(SEQ ID NO:12)** alone, or D477**K** mutant **(SEQ ID NO:19)** alone or in the presence of both D196**N** and D477**K** mutants, for either 25 minutes (*open bars*) or 1.5 hours (*solid bars*).

[0058] FIG. 12 is a sequence alignment of pmCS and pmHAS. The two *Pasteurella* GAG synthases are highly homologous. Identical residues are denoted with the *hyphen*. The numbering scheme corresponds to the slightly longer pmHAS sequence. The putative A1 (residues 161-267**; SEQ ID NO:72**) and A2 (residues 443-547**; SEQ ID NO:73**) domains correspond to regions important for hexosamine transferase or for glucuronic acid transferase activity, respectively ~~(33)~~. Most sequence differences are found in the amino-terminal half of the polypeptides.

[0060] FIG. 14 graphically depicts Sequence Similarity of pmHS1 **(SEQ ID NO:6)** with KfiA **(SEQ ID NO:63)** and KfiC **(SEQ ID NO:64)**. Elements of the *Pasteurella* heparosan synthase, HS1 (containing residues 91-240) and HS2 (containing residues 441-540) are very similar to portions of two proteins from the *E. coli* K5 capsular locus (A, residues 75-172 of KfiA; C, residues 262- 410 of KfiC) as shown by this modified Multalin alignment (~~ref. 21;~~ numbering scheme corresponds to the pmHS1 sequence). The HS1 and HS2 elements may be important for hexosamine transferase or for glucuronic acid transferase activities, respectively. (con, consensus symbols: asterisks, [K or R] and [S or T]; %, any one of F,Y,W; $, any one of L,M; !, any one of I,V; #, any one of E,D,Q,N).

[0061] FIG. 15(A-D) graphically depicts the alignment of the pmHS1 (two clones: A2 **(SEQ ID NO:6)**, B10 **(SEQ ID NO:70)**) with PmHS2 **(SEQ ID NO:8)**, KfiA **(SEQ ID NO:63)**, KfiC **(SEQ ID NO:64)**, and DcbF **(SEQ IDNO:61)**. pmHS1 is shown in various forms: HSA1 and HSA2 are the two putative domains of pmHS1; pORF = partial open reading frame which was obtained before complete sequence determined; recon = reconstructed open reading frame with sequence from multiple sources.

[0062] FIG. 16 depicts chimeric constructs of pm-EG **(SEQ ID NO:74)**, pm-FH **(SEQ ID NO:75)**, pm-IK **(SEQ ID NO:76)**, and pm-JL **(SEQ ID NO:77)**. PCR-overlap-extension was performed. Pm-EG contains residues 1-265 from pmHAS and residues 259-704 from pmCS and is a GlcUA-Tase. Pm-FH contains residues 1-258 from pmCS and residues 266-703 from pmHAS and is an active chondrotin synthase. Pm-IK contains residues 1-221 from pmHAS and residues 215-704 from pmCS and is a Glc-UA-Tase. Pm-JL contains residues 1-214 from pmCS and residues 222-703 from pmHAS and is an active HA synthase. The

switch of Gal-NAc-transferring activity into GlcNAc-transferring activity indicated that 222-265 of pmHAS and possibly the corresponding residues 215-258 of pmCS play critical role in the selectivity between binding and/or transferring of GalNAc and GlcNAc substrate.

[0063] FIG. 17 depicts a comparison of partial primary sequences of pmHAS and different pmCSs. Primary sequences of presumably chondroitin synthases from different Type F *Pasteruella multocida* were obtained by directly sequencing the products of colony-lysis PCR. The MULTALIN alignment indicates that most of the differences between pmHAS and pmCS are conserved among these independent strains. Residues that were substituted in site-mutagenesis studies were underlined. The mutant polypeptides contain a single or combination of different mutations, indicated by *star(s)*. None of these mutations changes the specificity of the original enzymes. **PmHAS, SEQ ID NO:78; PmCS, SEQ ID NO:79; Turkey, SEQ ID NO:80; Goose, SEQ ID NO:81; Sea-Lion, SEQ ID NO:82; Consensus, SEQ ID NO:83.**

[0064] FIG. 18 depicts chimeric constructs of $pmHAS^{1-221}$-$CS^{215-258}$-$HAS^{266-703}$ **(SEQ ID NO:84)** and $pmCS^{1-214}$-$HAS^{222-265}$-$CS^{258-704}$ **(SEQ ID NO:85)**. Pm-FH and pPm7A DNA were used to create $pmHAS^{1-221}$-$CS^{215-258}$-$HAS^{266-703}$. A very interesting result was that $pmCS^{1-214}$ -$HAS^{222-265}$ -$CS^{258-704}$ can transfer both GalNAc and GlcNAc to HA oligomer acceptor; this enzyme displays relaxed sugar specificity.

[0065] FIG. 19 depicts a summary of enzyme activities of chimeric proteins. The enzymes are drawn as bars. *Black* bars represent pmCS. *White* bars represent pmHAS. +, active; -, inactive. PmCHC represents $pmCS^{1-214}$-$HAS^{222-265}$ -$CS^{258-704}$ **(SEQ ID NO:85)**. The roles of the two domains are

confirmed and we have localized a 44-residue region critical for distinguishing C4 epimers of the hexosamine precursor.

[0106] Nucleic acid segments having HAS or CS or HS activity may be isolated by the methods described herein. The term "a sequence essentially as set forth in SEQ ID NO:X" means that the sequence substantially corresponds to a portion of SEQ ID NO:X and has relatively few amino acids or codons encoding amino acids which are not identical to, or a biologically functional equivalent of, the amino acids or codons encoding amino acids of SEQ ID NO:X. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein, as a gene having a sequence essentially as set forth in SEQ ID NO:X, and that is associated with the ability of prokaryotes to produce HA or a hyaluronic acid or chondroitin or heparin polymer *in vitro* or *in vivo*. In the above examples AX@ refers to either SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 69, 70 or 71 or any additional sequences set forth herein, such as the truncated or mutated versions of $pmHAS^{1-703}$ that are contained generally in SEQ ID NOS:~~10-60~~ **10-21 and 27-50**.

[0108] These references and countless others, indicate that one of ordinary skill in the art, given a nucleic acid sequence or an amino acid, could make substitutions and changes to the nucleic acid sequence without changing its functionality (specific examples of such changes are given hereinafter and are generally set forth in SEQ ID NOS:~~10-60~~ **10-21 and 27-50**). Also, a substituted nucleic acid segment may be highly identical and retain its enzymatic activity with regard to its unadulterated parent, and yet still fail to hybridize thereto. Additionally, the present application discloses 4 enzymes and numerous mutants of these enzymes that still retain at least 50% of the enzymatic activity of the unmutated parent enzyme – i.e., ½ of the dual action

transferase activity of the unadulterated parent. As such, variations of the sequences and enzymes that fall within the above-defined functional limitations have been disclosed and enabled. One of ordinary skill in the art, given the present specification, would be able to identify, isolate, create, and test DNA sequences and/or enzymes that produce natural or chimeric or hybrid GAG molecules. As such, the presently claimed and disclosed invention should not be regarded as being solely limited to the specific sequences disclosed herein.

Table IX

Mutants	**SEQ ID NO:**	**Enzyme Specific Activity**		
		HAS	**GlcNAc-Tase**	**GlcUA-Tase**
D477N	11	2	200%	2%
D477K	19	0.3	70%	2%
D477E	18	4	50%	4%
D196N	12	0.1	0%	74%
D196K	17	0.01	3%	100%
D196E	16	0.3	7%	60%

Specific activities of various $pmHAS^{1-703}$ mutants. Equivalent amounts of $pmHAS^{1-703}$ proteins (based on Western blot) were assayed. The specific activities (average of duplicate determinations) are indicated as the percentage of the wild-type sequence $pmHAS^{1-703}$ (set as 100%). The specific activities (picomoles of monosaccharide transfer/mg of protein/min) for wild-type enzyme in the three different assays were: HAS, 37; GlcNAc-Tase, 63; GlcUA-Tase, 76.

[0182] Briefly, the glycosyltransferase responsible for polymerizing the chondroitin backbone component of the capsular polysaccharide has also been molecularly cloned and was named pmCS (SEQ ID NO:4). The pmCS enzyme appears to be a close homolog of the pmHAS enzyme (FIG. 12). In pmHAS one domain, called A1 **(SEQ ID NO:72)**, is responsible for GlcNAc transfer and the other domain, called A2 **(SEQ ID NO:73)**, is responsible for GlcUA transfer.

Comparison of the pmHAS and the pmCS sequences reveals that the majority of the sequence differences exist in the A1 domain. The pmCS enzyme transfers a different hexosamine, GalNAc, thus this observation is consistent with the two-domain structure for pmHAS.

[0183] Mutant enzymes derived from the soluble $pmCS^{1-704}$ **(SEQ ID NO:26)** parental dual-action chondroitin synthase were also created with the ability to elongate HA or chondroitin-based oligosaccharides by adding a single β3-GalNAc monosaccharide to the non-reducing terminus. The mutants were formed by targeting the DXD motif in Domain A2 (also found in pmHAS) by site-directed mutagenesis (same general procedure as with pmHAS); the two aspartate (D) groups were converted into asparagine (N) residues forming the ANXN@ mutants. Several independent clones producing mutant $pmCS^{1-704}$ NXN enzyme were assayed individually for the ability to transfer [^{3}H]GalNAc to HA oligosaccharides using UDP-GalNAc in analogy to pmHAS transferring [^{3}H]GalNAc to HA oligosaccharides using UDP-GlcNAc as described hereinabove. The NXN mutants could transfer a single GalNAc sugar like the wild-type sequence $pmCS^{1-704}$ enzyme.

Table XII

Enzyme	SEQ ID NO:	Specific Activity		
		HAS	GlcNAc-Transferase	GlcUA-Transferase
D247N	**34**	<0.1	<0.1%	110%
D247K	**35**	<0.1	<0.1%	130%
D247E	**33**	<0.1	<0.1%	90%
D249K	**38**	<0.1	<0.1%	100%
D249E	**36**	<0.1	<0.1%	105%
D527K	**41**	<0.1	115%	<0.1%
D527E	**40**	<0.1	120%	0.1%
D529N	**43**	<0.1	230%	<0.1%
D529K	**44**	5%	360%	<0.1%
D529E	**42**	10%	110%	15%

Specific activities of the various pmHAS$^{1-703}$ DXD mutants. Equivalent amounts of pmHAS$^{1-703}$ proteins (based on Western blot) were assayed. The specific activities are indicated as the percentage of the wild-type sequence pmHAS$^{1-703}$ (set as 100%). The specific activities for wild-type enzyme in the three assays were 6-34 picomole of monosaccharide transfer /mg/min. The DXD motif of each domain is involved in HA polymerization.

Table XIII

Enzyme	SEQ ID NO:	Specific Activity			
		GlcNAc-Transferase		GlcUA-Transferase	
		Co^{2+}	Mg^{2+}	Co^{2+}	Mg^{2+}
D247N	34			15%	52%
D247K	35			1%	37%
D247E	33			9%	55%
D249N	37			14%	58%
D249K	38			10%	46%
D527E	40	87%	27%		
D529N	39	75%	59%		
Wt	71	77%	39%	18%	66%

Metal ion preference of the GlcNAc-transferases and the GlcA-transferase activities. Equivalent amounts of wild type pmHAS$^{1-703}$ protein (wt) or DXD mutants were assayed in the presence of 20 mM of Mn^{2+}, Co^{2+} or Mg^{2+}. The activities are indicated as the percentage of their activities in the presence of Mn^{2+} (set as 100%). Overall, Mn^{2+} is the best cofactor, but in its absence, the GlcNAc-transferase preferred Co^{2+} while the GlcUA-transferase preferred Mg^{2+}. The active sites of domain A1 and A2 are similar yet distinct.

Table XIV

Enzyme	SEQ ID NO:	Specific Activity		
		HAS	GlcNAc-Transferase	GlcUA-Transferase
D370N	49	<0.1	1%	80%
D370K	50	<0.1	2%	80%
D370E	48	1%	<0.1%	105%
E369H	47	<0.1	5%	130%
E369D	45	<0.1	1%	55%
E369Q	46	1%	1%	60%

Specific activities of the $pmHAS^{1-703}$ WGGED mutants. Equivalent amounts of $pmHAS^{1-703}$ proteins (based on Western blot) were assayed. The activities are indicated as the percentage of the wild type $pmHAS^{1-703}$ (100%). The WGGED motif is involved in the transfer of GlcNAc.

[0195] The chondroitin synthase, pCS, from Type F P. multocida is about 90% identical to pmHAS at the protein level. The majority of sequence differences exist in the vicinity of the domain A1 of pmHAS while their carboxyl-terminal halves are almost identical (described hereinabove). This is to be expected because the carboxyl-terminal half of pmHAS contains domain A2 which has the GlcUA-transferase active site. The pmCS also possesses two separate transferase sites with respect to pmCS, but the amino-terminal half is a GalNAc-transferase while the carboxyl-terminal half is a GlcUA-transferase. Thus, swapping the carboxyl-terminal GlcUA-transferase site between pmHAS and pmCS does not affect the sugar polymerizing activity. On the other hand, swapping of the amino-half of either pmHAS or pmCS changes the hexosamine transfer specificity. In order to test such "swapping@ abilities, domain swapping between pmHAS and pmCS was performed by the PCR-overlapping-extension method (as described in Horton et al., 1989, which is expressly incorporated herein by reference in its entirety).The active truncated versions of the synthases, $pmCS^{1-704}$ **(SEQ ID NO:26)** and $pmHAS^{1-703}$ **(SEQ ID NO:71)**, were used as the starting materials for the construction. Residues 427/428 of pmHAS and the equivalent site of pmCS, residues 420/421, were

chosen as the initial splicing site based on comparisons of the amino acid sequences of pmHAS, pmCS and other GlcNAc-transferases.

[0206] As stated hereinabove, *Pasteurella multocida* Type D, a causative agent of atrophic rhinitis in swine and pasteurellosis in other domestic animals, produces an extracellular polysaccharide capsule that is a putative virulence factor. It has been reported that the capsule of Type D was removed by treating microbes with heparin lyase III. A 617-residue enzyme, pmHS1 (SEQ ID NOS: ~~5~~ **6** and 70), and a 651-residue enzyme, PmHS2 (SEQ ID NO: 8), which are both authentic heparosan (unsulfated, unepimerized heparin) synthase enzymes have been molecularly cloned and are presently claimed and disclosed in copending U.S. Application Serial No. 10/142,143, incorporated herein previously by reference. Recombinant *Escherichia coli*-derived pmHS1 or PmHS2 catalyzes the polymerization of the monosaccharides from UDP-GlcNAc and UDP-GlcUA. Other structurally related sugar nucleotides do not substitute. Synthase activity was stimulated about 7- to 25-fold by the addition of an exogenous polymer acceptor. Molecules composed of ~500 to 3,000 sugar residues were produced *in vitro*. The polysaccharide was sensitive to the action of heparin lyase III but resistant to hyaluronan lyase. The sequence of pmHS1 enzyme is not very similar to the vertebrate heparin/heparan sulfate glycosyltransferases, EXT1/2 (SEQ ID NOS: 65/66), or to other *Pasteurella* glycosaminoglycan synthases that produce hyaluronan or chondroitin. Certain motifs do exist however, between the pmHS1, pmHS2, and KfiA (SEQ ID NO:~~65~~ **63**) and KfiC (SEQ ID NO:64) thereby leading to deduced amino acid motifs that are conserved throughout this class of GAG synthases for the production of heparin/heparosan. The pmHS1 and PmHS2 enzymes are the first microbial dual-action glycosyltransferase to be described that form a polysaccharide composed of β4GlcUA-α4GlcNAc disaccharide repeats. In contrast, heparosan

biosynthesis in *E. coli* K5 requires at least two separate polypeptides, KfiA and KfiC, to catalyze the same polymerization reaction.